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Plenary Paper

HEMATOPOIESIS AND STEM CELLS

Endothelial cells translate pathogen signals into G-CSF-driven emergency granulopoiesis

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Key Points

- ECs express *Tlr4* and *Myd88* and, after in vivo LPS or *E coli* stimulation, are the prime sources of G-CSF.
- ECs are sensors of systemically spread pathogens and subsequent drivers of BM emergency granulopoiesis.

Systemic bacterial infection induces a hematopoietic response program termed “emergency granulopoiesis” that is characterized by increased de novo bone marrow (BM) neutrophil production. How loss of local immune control and bacterial dissemination is sensed and subsequently translated into the switch from steady-state to emergency granulopoiesis is, however, unknown. Using tissue-specific myeloid differentiation primary response gene 88 (*Myd88*)-deficient mice and in vivo lipopolysaccharide (LPS) administration to model severe bacterial infection, we here show that endothelial cells (ECs) but not hematopoietic cells, hepatocytes, pericytes, or BM stromal cells, are essential cells for this process. Indeed, ECs from multiple tissues including BM express high levels of *Tlr4* and *Myd88* and are the primary source of granulocyte colony-stimulating factor (G-CSF), the key granulopoietic cytokine, after LPS challenge or infection with *Escherichia coli*. EC-intrinsic MYD88 signaling and subsequent G-CSF production by ECs

is required for myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors, increased colony-forming unit granulocyte activity in BM, and accelerated BM neutrophil generation after LPS stimulation. Thus, ECs catalyze the detection of systemic infection into demand-adapted granulopoiesis. (*Blood*. 2014;124(9):1393-1403)

Introduction

Granulocytes are generated from upstream bone marrow (BM) precursors under the control of various myeloid cytokines, most importantly granulocyte colony-stimulating factor (G-CSF).¹ Among granulocytes, neutrophils are the dominant cell type in BM, peripheral blood (PB), and tissues in the steady state and during bacterial infection. Neutrophils serve as key first line of defense cells of the innate immune system.² To exert their critical function, neutrophils are recruited to respective sites of infection.² Although some neutrophils are able to reenter the vasculature,³⁻⁵ the majority of cells are consumed and undergo cell death in the inflamed tissue as a consequence of launching their antimicrobial defense mechanisms.² In locally poorly controlled and consequently systemically spread microbial infection, neutrophils are in high demand and need to be regenerated in large numbers. Hence, because of their already short half-life in the steady state, ranging from a few hours to a few days,⁶ during severe systemic infection, steady-state granulopoiesis is switched to “emergency granulopoiesis” (ie, massively enhanced de novo neutrophil production in BM).^{7,8} Thus, besides efficient neutrophil recruitment to sites of infection and microbicidal neutrophil effector functions,² the third critical component of a protective innate immune response is the ability of the hematopoietic system to launch the emergency granulopoiesis program.

A prerequisite for the initiation of emergency granulopoiesis is sensing of pathogen dissemination. Pattern-recognition receptors

such as Toll-like receptors (TLRs) recognize respective conserved pathogen-associated molecular patterns as diverse as proteins, polysaccharides, and nucleic acids.⁹ TLRs can be found on the cell surface as well as within endosomal compartments, and TLR signal transduction occurs via adaptor molecules such as MYD88 and TRIF, leading to transcription of genes involved in the host response toward invading pathogens.¹⁰ TLR expression and function has been best characterized in mature immune effector cells.⁹ In addition, accumulating evidence has revealed that nonhematopoietic cells such as bladder epithelial cells,¹¹ mesenchymal stromal cells,^{12,13} and endothelial cells (ECs)^{14,15} also express TLRs, thereby critically contributing to innate immune responses. Furthermore, it has been demonstrated that hematopoietic stem and progenitor cells (HSPCs) express some TLRs and directly respond to pathogen-associated molecular patterns with increased myelopoiesis and directed migration to inflamed sites.¹⁶⁻²² However, we have recently shown that direct pathogen sensing by HSPCs does not play an essential role in the immediate lipopolysaccharide (LPS)-induced emergency granulopoiesis response. By contrast, TLR4 agonist sensing by non-hematopoietic cells followed by granulopoietic growth factor release, primarily G-CSF, is the main route for the initiation of emergency granulopoiesis.²³

We address here the fundamental question of which cell type out of the vast plethora of putative nonhematopoietic cells and tissues is

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the key sensor of systemically spread pathogens, specifically LPS and *Escherichia coli*, and how this sensing is translated into emergency granulopoiesis.

Methods

Mice

C57BL/6J (CD45.2⁺), B6.SJL-*Ptprca*^a *Pepc*^b/BoyJ (CD45.1⁺), *Myd88*^{-/-}, *Tlr4*^{-/-}, *Trif*^{-/-}, *LysM-Cre*, *Nes-Cre*, *Pdgfrb-Cre*, *Alb-Cre*, *Tie2-Cre*, *Myd88*^{fl/fl} (B6.129P2(SJL)-*Myd88*^{tm1Defr}/J), and *loxP-GFP* (B6.Cg-*Gt(ROSA)26Sor*^{tm6(CAGZsGreen1)Hze}/J) mice were used in this study. All animals were maintained at the University Hospital Zurich animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments and procedures were approved by the Veterinäramt des Kantons, Zurich, Switzerland.

LPS injections, *E coli* infection, and G-CSF injections

Mice received 2 intraperitoneal injections with 35 μ g ultrapure LPS from *E coli* 0111:B4 (InvivoGen) 48 hours apart and were analyzed 24 hours after the second injection. For some experiments, mice were administered 20 μ g LPS 3 times (at 0, 12, and 20 hours) and were analyzed at time point 24 hours. Alternatively, mice were intraperitoneally (IP)-injected with 0.5 to 4.5 $\times 10^8$ *E coli* and analyzed 48 hours later. Some mice were IP-injected with 250 μ g/kg body weight human G-CSF (Filgrastim; Amgen) 6 times in a 12-hour interval and analyzed as LPS-injected mice.

Flow cytometry

The following antibodies (all from eBiosciences, unless otherwise stated) were used to assess mature BM and PB cell populations: anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), anti-Ly6-G (1A8; Biolegend), anti-Ly6-C (HK1.4), anti-B220 (RA3-6B2), and anti-CD3e (145-2C11). For fluorescence-activated cell sorter (FACS) analysis of HSPCs, the following antibodies were used: anti-CD3e (145-2C11), anti-CD4 (GK1.5), anti-CD8 α (53-6.7), anti-B220 (RA3-6B2), anti-CD19 (MB19-1), anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), anti-Ter119 (Ter-119), anti-IL7R α (A7R34), anti-cKit (2B8), anti-Sca1 (D7), anti-CD34 (RAM34), anti-Fcgr (93), and anti-NK1.1 (PK136; Becton Dickinson).

ECs were stained with anti-CD45 (30-F11), anti-TER119 (Ter-119), and anti-CD31 (390). In some experiments, anti-VE-cadherin (BV13), anti-Sca1 (E13-161.7; BD Biosciences), anti-VCAM1 (429), and anti-CD105 (MJ7/18) antibodies were used.

Classical and plasmacytoid dendritic cells were stained using anti-CD3e (145-2C11), anti-CD19 (MB19-1), anti-NK1.1 (PK136; Becton Dickinson), anti-CD45RA (14.8; BD Biosciences), anti-CD11c (N418), and anti-MHCII (M5/114.15.2).

Hoechst33342 (Life Technologies) was used to exclude dead cells. Cells were analyzed or sorted on a FACS Canto II or FACS Aria III flow cytometer (BD Biosciences), respectively. Data were analyzed by FlowJo (Tree Star) software.

BrdU incorporation assay

Mice were injected with phosphate-buffered saline (PBS) or LPS according to the scheme depicted in Figure 5A. Twelve hours before analysis, mice received 1 single IP injection of 2 mg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich). In addition, BrdU at a final concentration of 0.8 mg/mL as well as 5% glucose were added to the drinking water. Cells were isolated and further processed according to the manufacturer's protocol (BrdU Flow Kit; BD Pharmingen).

Colony-forming unit (CFU) assay

After in vivo PBS or LPS stimulation according to the scheme depicted in Figure 1A, 1×10^4 red blood cell-depleted whole BM cells were plated in methylcellulose (Methocult M3234; StemCell Technologies) mixed with

Iscove's modified Dulbecco's medium (30% fetal calf serum, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol) with the following factors added: mIL3 (10 ng/mL), hIL6 (10 ng/mL), hIL11 (10 ng/mL), mFLT3-Ligand (10 ng/mL), mSCF (10 ng/mL), mGM-CSF (10 ng/mL), huTPO (50 ng/mL), and huEPO (4 U/mL) (all R&D Systems). Colonies were scored after 8 days of in vitro culture.

Quantitative reverse-transcription PCR

ECs were sorted directly into lysis buffer belonging to RNeasy Micro Kit (QIAGEN). Total RNA extraction was carried out according to the manufacturer's protocol and subsequently subjected to reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR green reagent (Applied Biosystems) and samples were run on a 7500 FAST real-time PCR thermal cycler (Applied Biosystems). *Actb* was used to normalize the RNA content of samples.

Enzyme-linked immunosorbent assay

PB was obtained from mice postmortem by cardiac puncture using heparinized syringes and centrifuged to obtain plasma. G-CSF plasma levels were measured using Quantikine ELISA kits according to the manufacturer's instructions (R&D Systems).

Equations and statistical analyses

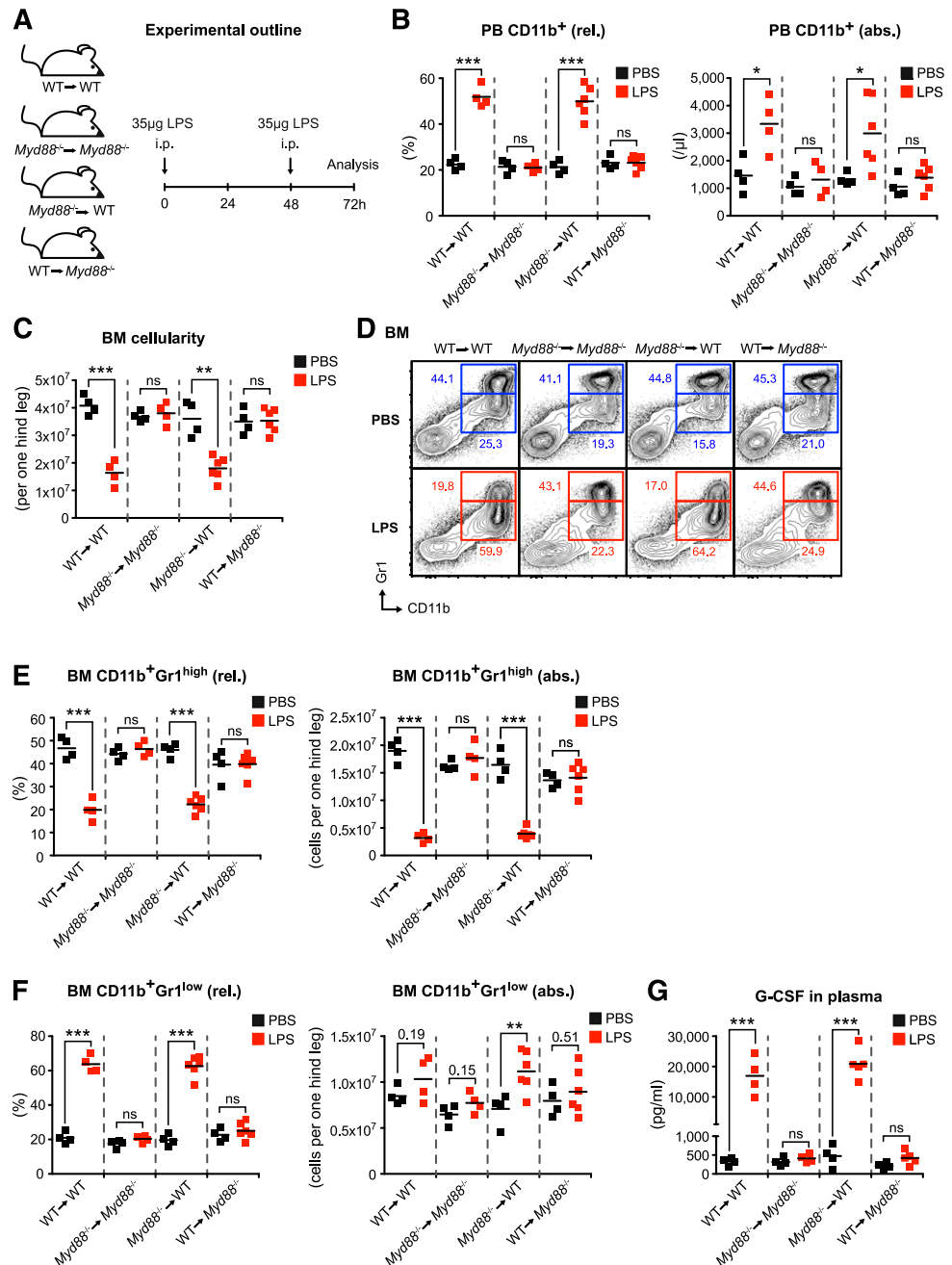
Significance of differences was analyzed using Student *t* test. A difference between experimental groups was considered significant when the *P* value was <.05. All statistical analyses were calculated with Prism software (GraphPad Software, version 5.01).

Results

LPS-induced emergency granulopoiesis requires intact MYD88-mediated TLR signaling in nonhematopoietic cells

To examine whether MYD88 expression within hematopoietic or nonhematopoietic cells is required for LPS-induced emergency granulopoiesis, we generated BM chimeric mice with MYD88 expression restricted to either the hematopoietic (wild-type [WT] \rightarrow *Myd88*^{-/-}) or the nonhematopoietic cellular compartment (*Myd88*^{-/-} \rightarrow WT) as well as respective control mice (WT \rightarrow WT and *Myd88*^{-/-} \rightarrow *Myd88*^{-/-}) (Figure 1A). We IP-injected chimeric mice with high doses of LPS to mimic severe systemic infection (Figure 1A) and analyzed typical surrogate hallmarks of emergency granulopoiesis. Although absolute leukocyte counts did not change significantly in PB upon LPS stimulation (data not shown), the frequency and absolute numbers of PB CD11b⁺ cells significantly increased in WT \rightarrow WT mice (Figure 1B). This response was completely absent in *Myd88*^{-/-} \rightarrow *Myd88*^{-/-} mice, indicating that LPS-induced elevation in PB CD11b⁺ cells depends on MYD88 signaling. Strikingly, although WT \rightarrow *Myd88*^{-/-} mice were also completely nonresponsive toward LPS, *Myd88*^{-/-} \rightarrow WT mice showed a normal LPS response (Figure 1B). Next, we analyzed the BM of chimeric mice after LPS treatment. We found that total BM cell numbers are significantly decreased upon LPS stimulation in WT \rightarrow WT mice (Figure 1C). The reduction of BM cellularity is mediated in a MYD88-dependent manner because *Myd88*^{-/-} \rightarrow *Myd88*^{-/-} mice did not show this response. This depends on MYD88 expression within nonhematopoietic cells because *Myd88*^{-/-} \rightarrow WT mice responded normally but WT \rightarrow *Myd88*^{-/-} mice lacked this response (Figure 1C). A characteristic feature of emergency granulopoiesis is a significant, relative, and absolute decrease in BM CD11b⁺ Gr1^{high} mature neutrophils (Figure 1D-E). This response is paralleled by a significant relative increase in BM CD11b⁺ Gr1^{low} immature neutrophils²³⁻²⁵ and

Figure 1. LPS-induced emergency granulopoiesis requires intact MYD88-mediated TLR signaling in nonhematopoietic cells. (A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis in mice with MYD88 expression restricted to hematopoietic ($WT \rightarrow Myd88^{-/-}$) or nonhematopoietic ($Myd88^{-/-} \rightarrow WT$) cells, and respective control mice ($WT \rightarrow WT$ and $Myd88^{-/-} \rightarrow Myd88^{-/-}$). (B) Frequency and absolute number of PB CD11b⁺ cells, and (C) BM cellularity after LPS stimulation in BM chimeric and control mice. (D) Representative FACS profile showing characteristic changes in BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils after LPS stimulation. (E) Frequencies and absolute numbers of BM CD11b⁺Gr1^{high} mature, (F) BM CD11b⁺Gr1^{low} immature neutrophils, and (G) plasma G-CSF levels in reciprocal $Myd88^{-/-}$ BM chimeras upon systemic LPS injection. Black squares, PBS-injected mice; red squares, LPS-injected mice; rel., relative; abs., absolute. Data from 2 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (**P* < .05, ***P* < .01, ****P* < .001; ns, nonsignificant).



a trend toward an absolute increase in BM CD11b⁺Gr1^{low} immature neutrophils. These reciprocal changes in BM CD11b⁺Gr1^{high} mature neutrophils and BM CD11b⁺Gr1^{low} immature neutrophils after LPS injection require intact MYD88 signaling in nonhematopoietic cells (Figure 1D-F).

Although various cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor, interleukin-6, and G-CSF have the capacity to stimulate both steady-state as well as emergency granulopoiesis, none of these cytokines is absolutely essential.^{1,26-29} However, G-CSF plays an outstanding role in these processes as demonstrated in studies of *G-csf*^{-/-} and *G-csfr*^{-/-} mice showing hematopoietic defects in steady state, and most importantly, upon infection.^{1,30,31} Notably, administration of recombinant G-CSF is routinely used to treat iatrogenic³² and congenital³³ neutropenia. Furthermore, G-CSF

alone is sufficient to induce a response indistinguishable from LPS-induced emergency granulopoiesis.^{23,34} We therefore assessed G-CSF plasma levels in BM chimeric mice after LPS stimulation. We found highly significant increases in plasma G-CSF levels in $WT \rightarrow WT$ and $Myd88^{-/-} \rightarrow WT$ mice (Figure 1G). By contrast, $Myd88^{-/-} \rightarrow Myd88^{-/-}$ and $WT \rightarrow Myd88^{-/-}$ mice did not show elevated plasma G-CSF levels. Thus, LPS-triggered G-CSF release requires intact *Myd88* within nonhematopoietic cells.

To corroborate our findings on the importance of nonhematopoietic cells for the initiation of emergency granulopoiesis and to further rule out a role for tissue-resident macrophages for this process, we generated *LysM-Cre;Myd88^{fl/fl}* mice with a *Myd88* deficiency restricted to myeloid cells including macrophages.³⁵ In line with the results on hematopoietic chimeric animals, we observed that

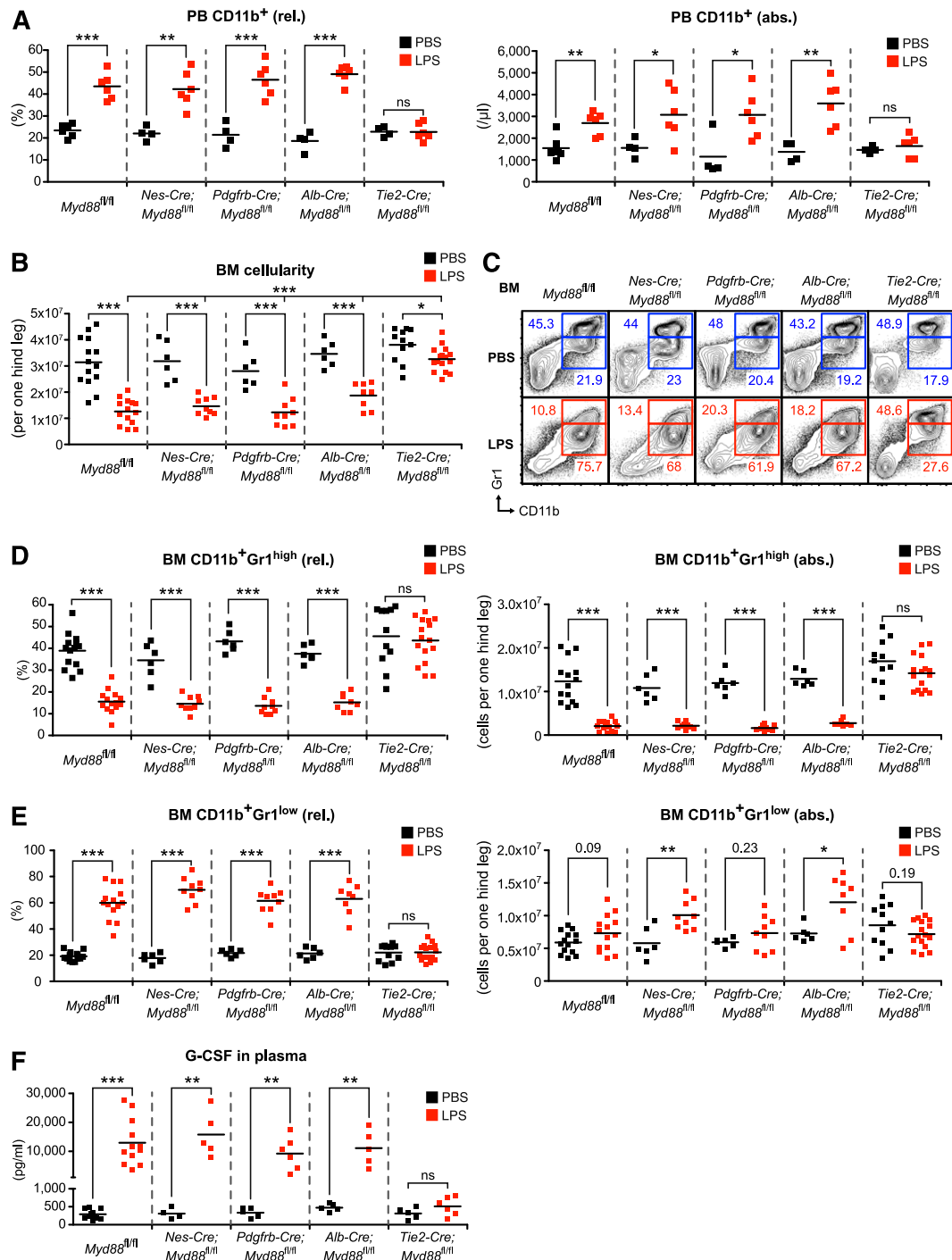


Figure 2. LPS-induced emergency granulopoiesis is abrogated in *Tie2-Cre;Myd88^{fl/fl}* mice. (A) Frequency and absolute number of PB CD11b⁺ cells after LPS stimulation in control *Myd88^{fl/fl}*, and experimental *Nes-Cre;Myd88^{fl/fl}*, *Pdgfrb-Cre;Myd88^{fl/fl}*, *Alb-Cre;Myd88^{fl/fl}*, and *Tie2-Cre;Myd88^{fl/fl}* mice. (B) BM cellularity after LPS stimulation in control *Myd88^{fl/fl}*, and experimental *Nes-Cre;Myd88^{fl/fl}*, *Pdgfrb-Cre;Myd88^{fl/fl}*, *Alb-Cre;Myd88^{fl/fl}*, and *Tie2-Cre;Myd88^{fl/fl}* mice. (C) Representative FACS profile showing characteristic LPS-induced changes in BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils in control *Myd88^{fl/fl}*, and experimental *Nes-Cre;Myd88^{fl/fl}*, *Pdgfrb-Cre;Myd88^{fl/fl}*, *Alb-Cre;Myd88^{fl/fl}*, and *Tie2-Cre;Myd88^{fl/fl}* mice. (D) Frequencies and absolute numbers of BM CD11b⁺Gr1^{high} mature and (E) BM CD11b⁺Gr1^{low} immature neutrophils, and (F) plasma G-CSF levels in the different sets of tissue-specific *Myd88^{fl/fl}* mice after systemic LPS injection. Black squares, PBS-injected mice; red squares, LPS-injected mice; rel., relative; abs., absolute. Data from at least 3 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (**P* < .05, ***P* < .01, ****P* < .001).

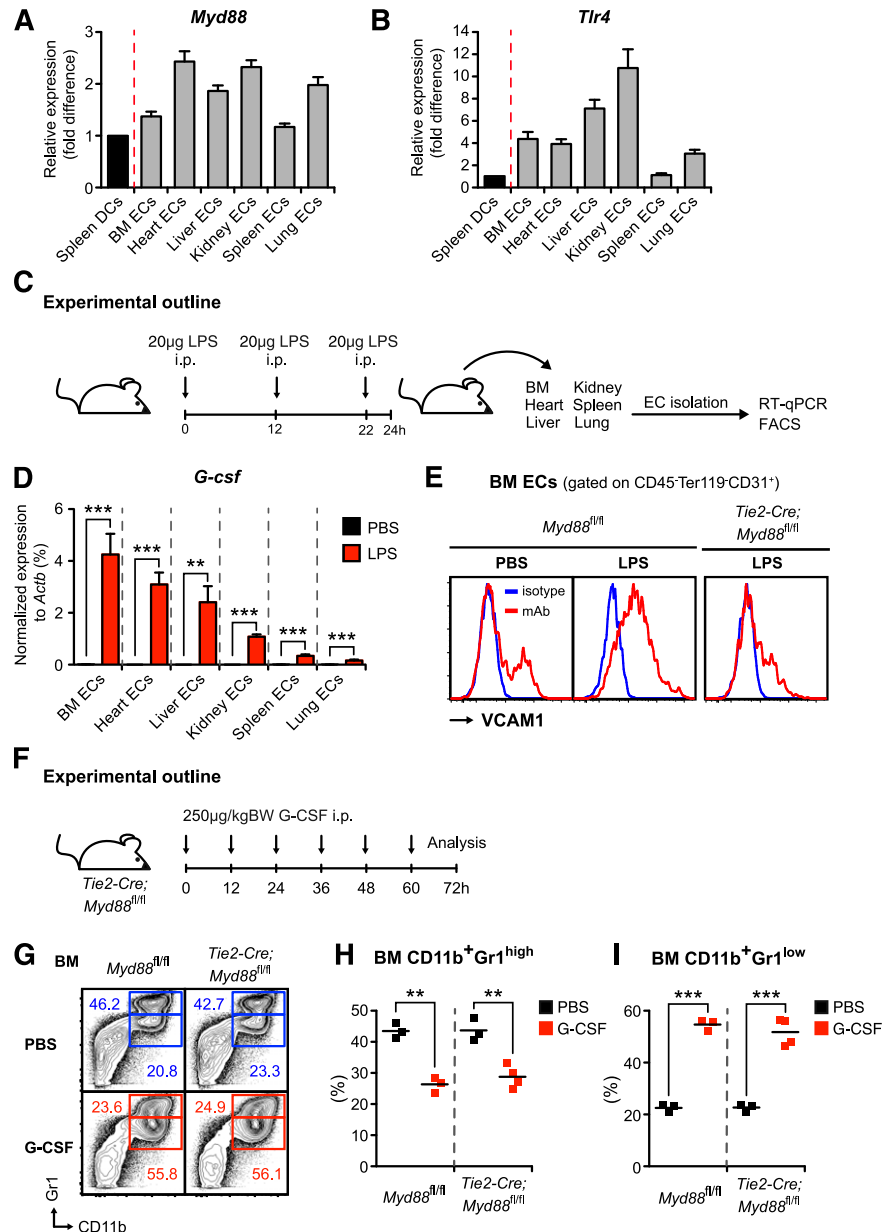
LysM-Cre;Myd88^{fl/fl} mice are fully capable to launch emergency granulopoiesis (supplemental Figure 1, available on the Blood Web site).

Altogether, our results on MYD88 and previous data on TLR4²³ unambiguously show that LPS-induced emergency granulopoiesis is initiated through TLR agonist sensing and MYD88-dependent signaling by nonhematopoietic cells.

LPS-induced emergency granulopoiesis is abrogated in *Tie2-Cre;Myd88^{fl/fl}* mice

To unravel the identity of the nonhematopoietic cell type critical for emergency granulopoiesis, we used *Cre-loxP* recombination technology to delete *Myd88* from various candidate nonhematopoietic cell

Figure 3. Endothelial cells from various organs express high levels of *Myd88* and *Tlr4*, respond to LPS challenge with strong upregulation of VCAM-1 and *G-CSF* in vivo, and exogenous G-CSF administration rescues emergency granulopoiesis in *Tie2-Cre;Myd88^{fl/fl}* mice. (A) *Myd88* and (B) *Tlr4* expression were assessed by quantitative reverse-transcription PCR in CD45⁺Ter119⁺CD31⁺ ECs isolated from BM, heart, liver, kidney, spleen, and lung (gray bars) compared with spleen DCs (black bars), ie, pooled classical dendritic cells (CD3e⁺CD19⁺NK1.1⁺CD11c^{high}CD45RA⁺MHCII⁺) and plasmacytoid dendritic cells (CD3e⁺CD19⁺NK1.1⁺CD11c⁺CD45RA⁺MHCII^{high}). All cells were isolated from steady-state mice. (C) Graphical scheme depicting experimental outline to assess in vivo LPS responsiveness of ECs that were flow-cytometrically sorted from BM, heart, liver, kidney, spleen, and lung of PBS- and LPS-injected mice, respectively. (D) Comparative *G-CSF* transcript levels normalized to *Actb* in ECs from BM, heart, liver, kidney, spleen, and lung of LPS-injected (red bars) vs PBS-injected (black bars) wild-type mice. (E) Representative FACS analysis depicting cell-surface expression of VCAM1 (red lines) on BM ECs in steady state as well as LPS-injected control *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. Isotype control shown as blue line. (F) Graphical scheme showing experimental outline to assess G-CSF effects in vivo. (G) Representative FACS profile showing characteristic G-CSF-induced changes in BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils in control *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. (H) Frequencies of BM CD11b⁺Gr1^{high} mature and (I) BM CD11b⁺Gr1^{low} immature neutrophils in PBS- and G-CSF-injected control *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. All data represent mean \pm standard deviation from 2 or 3 independent experiments. Two-tailed Student *t* tests were used to assess statistical significance (***P* < .01, ****P* < .001).



populations to study their relative contribution to pathogen sensing (supplemental Table 1). To achieve *Myd88* deletion in cells of the BM microenvironment (*Nestin*⁺ mesenchymal stem cells and perivascular stromal cells), we used *Nes-Cre*³⁶⁻³⁸ and *Pdgfrb-Cre*³⁹ mice. Cells belonging to the vasculature (ECs and pericytes) were targeted using *Tie2-Cre*⁴⁰ and *Pdgfrb-Cre*³⁹ mice, respectively. We also employed *Alb-Cre*⁴¹ mice to target hepatocytes as a candidate parenchymal organ cell type. First, we assessed the LPS response in PB of control and tissue-specific *Myd88*^{-/-} mice. We observed that the frequency and absolute numbers of PB CD11b⁺ cells significantly increased in LPS-injected *Myd88^{fl/fl}* control, *Nes-Cre;Myd88^{fl/fl}*, *Pdgfrb-Cre;Myd88^{fl/fl}*, and *Alb-Cre;Myd88^{fl/fl}* mice, whereas *Tie2-Cre;Myd88^{fl/fl}* mice lacked the response (Figure 2A). The majority of PB CD11b⁺ cells could be identified as neutrophils based on coexpression of Ly6-G, whereas PB CD11b⁺Ly6-C^{high} inflammatory monocytes showed a relative decrease but overall stable absolute numbers (supplemental Figure 2).

Next, we investigated the BM response toward LPS. Not only did we observe a decrease in BM cellularity in LPS-injected *Myd88^{fl/fl}* control, *Nes-Cre;Myd88^{fl/fl}*, *Pdgfrb-Cre;Myd88^{fl/fl}*, and *Alb-Cre;Myd88^{fl/fl}* mice

(Figure 2B), we also revealed a reduction in the frequency and absolute number of BM CD11b⁺Gr1^{high} mature neutrophils (Figure 2C-D). Although the percentage of BM CD11b⁺Gr1^{low} immature neutrophils was highly significantly increased, there was only a minor increase in absolute numbers that, likely because of the higher variation in BM cellularity, did not always yield statistical significance (Figure 2C,E). Strikingly, *Tie2-Cre;Myd88^{fl/fl}* mice were nonresponsive toward systemic LPS injection, with the exception of a small but significant decrease in BM cellularity that was, however, significantly reduced in magnitude compared with the other groups of mice (Figure 2B-E). In line with these findings, G-CSF levels increased massively, up to 100-fold, in control and respective tissue-specific *Myd88*^{-/-} mice with the exception of *Tie2-Cre;Myd88^{fl/fl}* mice that lacked a significant rise in plasma G-CSF concentration (Figure 2F).

ECs express high levels of *Myd88* and *Tlr4* and respond to LPS challenge with strong upregulation of VCAM-1 and *G-CSF* in vivo

Our findings suggested that ECs, which are targeted in *Tie2-Cre* mice, are the cells within the nonhematopoietic compartment responsible

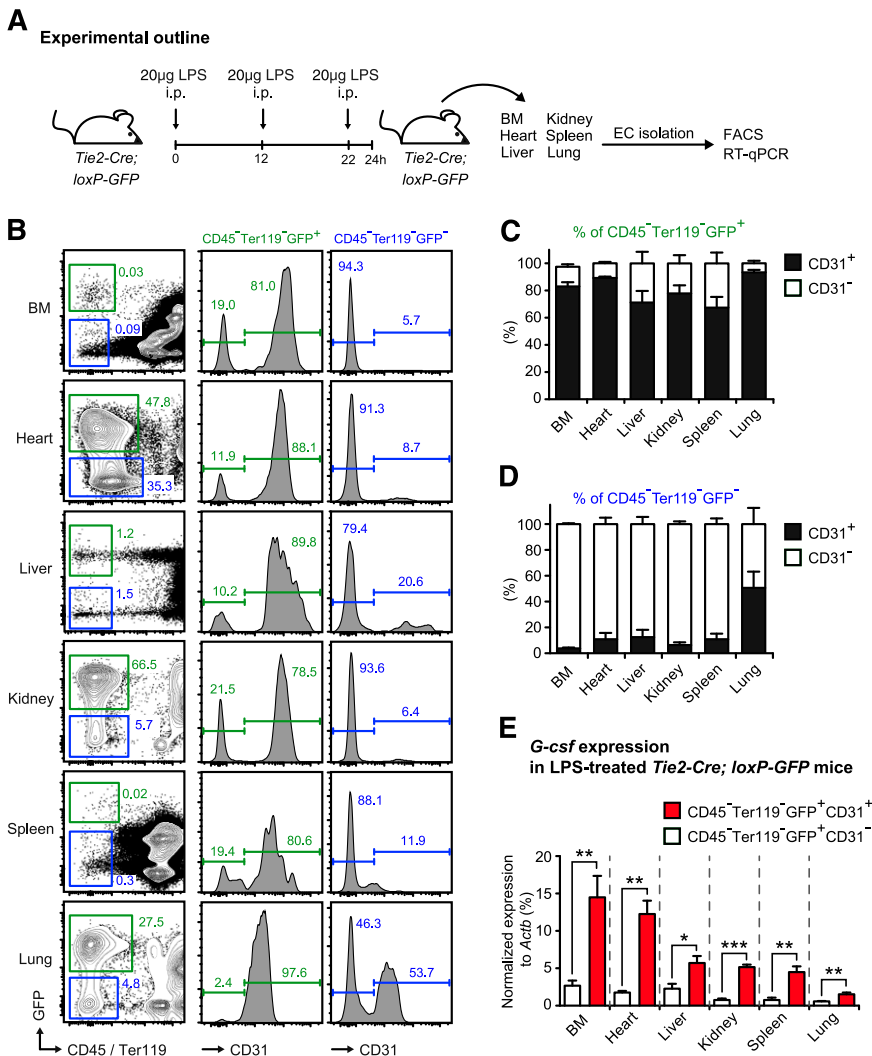


Figure 4. Endothelial cells are effectively targeted in *Tie2-Cre-loxP-GFP* reporter mice and are the main source of *G-CSF* after in vivo LPS stimulation.

(A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis and to assess *G-CSF* expression in sorted $CD45^{+}Ter119^{-}GFP^{+}$ ECs and $CD45^{+}Ter119^{-}GFP^{+}CD31^{-}$ non-ECs isolated from different organs of *Tie2-Cre-loxP-GFP* reporter mice. (B) Representative FACS profile of GFP and CD31 expression within nonhematopoietic cells ($CD45^{+}Ter119^{-}$) isolated from BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (C) Percentages of $CD31^{+}$ (black bars) and $CD31^{-}$ (white bars) cells within the $CD45^{+}Ter119^{-}GFP^{+}$ cell population in BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (D) Percentages of $CD31^{+}$ (black bars) and $CD31^{-}$ (white bars) cells within the $CD45^{+}Ter119^{-}GFP^{-}$ cell population in BM, heart, liver, kidney, spleen, and lung of *Tie2-Cre-loxP-GFP* reporter mice. (E) Comparative *G-CSF* transcript levels normalized to *Actb* in sorted $CD45^{+}Ter119^{-}GFP^{+}CD31^{+}$ ECs (red bars) vs $CD45^{+}Ter119^{-}GFP^{+}CD31^{-}$ non-ECs (white bars) isolated from BM, heart, liver, kidney, spleen, and lung of LPS-injected *Tie2-Cre-loxP-GFP* reporter mice. All data represent mean \pm standard deviation from 2 independent experiments ($n = 5$ mice). Two-tailed Student *t* tests were used to assess statistical significance (* $P < .05$, ** $P < .01$, *** $P < .001$).

for sensing LPS, translating this signal into G-CSF release, and consequently initiating emergency granulopoiesis.

To assess expression of *Tlr4* and *Myd88* in ECs, we isolated $CD45^{+}Ter119^{-}CD31^{+}$ ECs from BM, heart, lung, liver, spleen, and kidney and compared these with pooled spleen classical and plasmacytoid dendritic cells with defined TLR expression patterns.⁴² No immunophenotypical differences were observed between ECs from different organs with respect to the endothelial markers VE-cadherin, CD105, CD34, and Sca1 (data not shown). *Myd88* and *Tlr4* were abundantly expressed in ECs at similar or higher levels compared with dendritic cells, ie, cell populations known for their TLR expression profile (Figure 3A-B).²⁰

To analyze the functional relevance of *Tlr4* and *Myd88* expression in ECs, we injected mice with LPS or PBS (Figure 3C); isolated ECs from BM, heart, lung, liver, spleen, and kidney; and assessed *G-CSF* transcripts by quantitative real-time PCR. Although in the PBS-injected steady-state mice, *G-CSF* was only detectable at very low levels, LPS injection resulted in a strong upregulation of *G-CSF* (up to several hundred-fold) in ECs from all organs analyzed (Figure 3D). Interestingly, *G-CSF* induction was relatively highest in ECs from BM (ie, the primary site of hematopoiesis). However, it needs to be emphasized that this does not necessarily imply that absolute G-CSF amounts produced are also highest in this tissue. Transcription and protein production do not always correlate, and quantity of protein produced in a given organ will also depend on frequency of producing cells at site.

To test whether there might be heterogeneity in the responsiveness toward LPS within different sections of the vasculature, we analyzed the activation status of ECs upon systemic LPS stimulation by measuring VCAM1 cell-surface expression.⁴³ However, we did not detect differences between ECs isolated from different tissues and, importantly and in line with the observed nonresponsiveness, VCAM1 upregulation on ECs was abrogated in *Tie2-Cre;Myd88^{fl/fl}* mice (Figure 3E and data not shown).

To confirm that disrupted MYD88 signaling in *Tie2-Cre;Myd88^{fl/fl}* mice does not affect G-CSF signal transduction and consequently impairs emergency granulopoiesis, we injected *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice with recombinant human G-CSF (Figure 3F). In accordance with our previously published data on G-CSF effects in *Tlr4^{-/-}* mice,²³ we observed that G-CSF alone without an additional inflammatory stimulus is sufficient to accurately mimic emergency granulopoiesis (Figure 3G-I and data not shown).

Given the possibility of insufficient tissue specificity of *Cre-loxP* technology, we generated *Tie2-Cre-loxP-GFP* reporter mice to study the effectiveness and specificity of EC targeting. To this end, we isolated nonhematopoietic cells from BM, heart, liver, kidney, spleen, and lung and analyzed both GFP and expression of the EC marker CD31 by FACS (Figure 4A-D). In accordance with previously published reports,³⁷ we found that the vast majority of ECs (approximately 90%) were efficaciously targeted in *Tie2-Cre-loxP-GFP* reporter mice. This pattern was robustly observed in all tissues analyzed, except

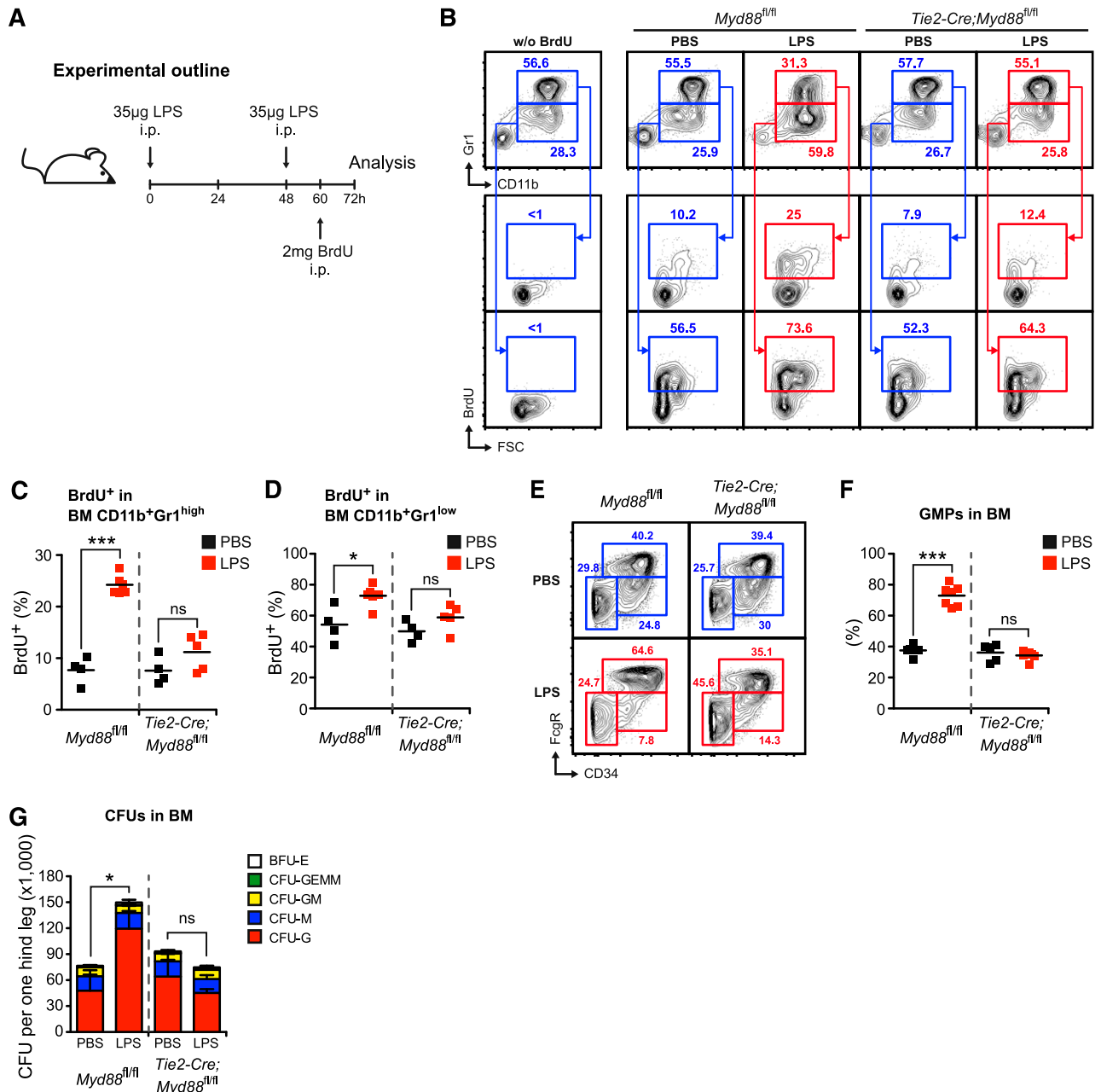


Figure 5. Endothelial cell-intrinsic MYD88 signaling is required to stimulate accelerated neutrophil production, myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors (GMPs), and increased CFU-G activity in vivo. (A) Graphical scheme depicting experimental outline to induce LPS-induced emergency granulopoiesis and to assess BrdU incorporation. (B) Representative FACS profile showing BrdU incorporation in in BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils in control *Myd88^{fl/fl}*, and *Tie2-Cre;Myd88^{fl/fl}* mice during steady-state and LPS-induced emergency granulopoiesis. (C) Frequencies of BrdU⁺ BM CD11b⁺Gr1^{high} mature and (D) BrdU⁺ BM CD11b⁺Gr1^{low} immature neutrophils in PBS- or LPS-injected *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. (E) Representative FACS profile showing myeloerythroid progenitors in control *Myd88^{fl/fl}*, and *Tie2-Cre;Myd88^{fl/fl}* mice during steady-state and LPS-induced emergency granulopoiesis. (F) Frequencies of Lin[−]cKit⁺Sca1[−]FcgR⁺CD34⁺ GMPs in PBS- or LPS-injected *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. (G) Absolute CFU numbers per 1 hind leg in control *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice during steady-state and LPS-induced emergency granulopoiesis (CFU-G, CFU granulocyte; CFU-M, CFU macrophage; CFU-GM, CFU granulocyte/macrophage; CFU-GEMM, CFU granulocyte/erythrocyte/macrophage/megakaryocyte; BFCU-E, burst-forming unit erythrocyte). Black squares, PBS-injected mice; red squares, LPS-injected mice. Data from 2 independent experiments are shown. Two-tailed Student *t* tests were used to assess statistical significance (**P* < .05, ****P* < .001).

for the lung, where we found a higher fraction of CD31⁺ cells within the CD45[−]Ter119[−]GFP[−] fraction (Figure 4B-D). Furthermore, we observed that about 20% (with some variations between the different tissues) of CD45[−]Ter119[−]GFP⁺ cells in *Tie2-Cre-loxP-GFP* reporter mice do not express the EC marker CD31 and thus, presumably, are not ECs (Figure 4B-C).

To evaluate the role of these CD45[−]Ter119[−]GFP⁺CD31[−] cells for the initiation of emergency granulopoiesis, we injected *Tie2-Cre-*

loxP-GFP reporter mice with LPS, sorted the CD31⁺ EC and CD31[−] non-EC fractions of the CD45[−]Ter119[−]GFP⁺ nonhematopoietic compartment and compared *G-csf* expression by quantitative reverse-transcription PCR as a surrogate marker for the ability of the respective cell type to stimulate emergency granulopoiesis (Figure 4A). There was no difference in steady-state *G-csf* expression between both cell fractions (data not shown), whereas after LPS injection, *G-csf* expression in CD45[−]Ter119[−]GFP⁺CD31⁺ ECs was always

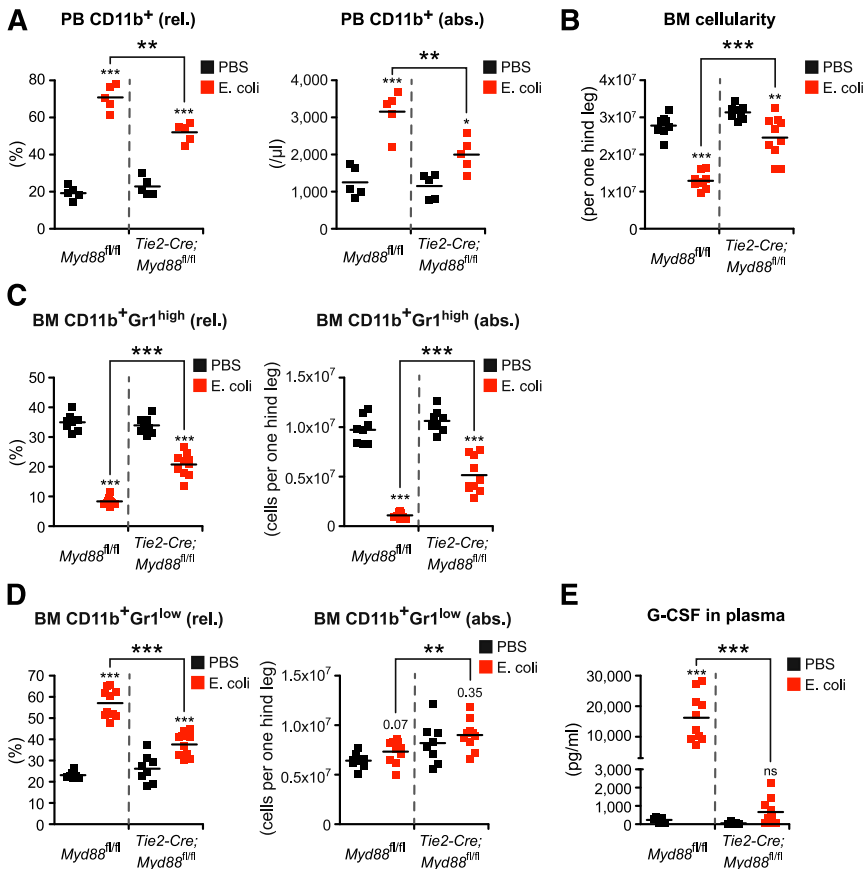


Figure 6. Endothelial cell-intrinsic MYD88 signaling is required to efficiently stimulate emergency granulopoiesis during systemic *E. coli* infection. (A) Frequency and absolute number of PB CD11b⁺ cells, (B) BM cellularity, (C) frequencies and absolute numbers of BM CD11b⁺Gr1^{high} mature and (D) BM CD11b⁺Gr1^{low} immature neutrophils, and (E) plasma G-CSF levels in *Myd88^{fl/fl}* mice and *Tie2-Cre;Myd88^{fl/fl}* mice in steady-state (black squares) and after infection with 4.5×10^8 *E. coli* CFU (red squares) given IP. Data from 2 independent experiments are shown. rel., relative; abs., absolute. Two-tailed Student *t* test was used to assess statistical significance (***P* < .001, ****P* < .001).

significantly higher (2- to 5-fold) than in the CD45⁺Ter119⁺GFP⁺CD31⁺ non-EC fraction in all organs analyzed (Figure 4E). Thus, given the 4-fold higher numbers of CD45⁺Ter119⁺GFP⁺CD31⁺ ECs vs CD45⁺Ter119⁺GFP⁺CD31⁺ non-ECs and the 2- to 5-fold higher amount of *G-CSF* expression in CD45⁺Ter119⁺GFP⁺CD31⁺ ECs vs CD45⁺Ter119⁺GFP⁺CD31⁺ non-ECs, we infer that most (>95%) of G-CSF derived from ECs and, importantly, *Tie2-Cre* mice, are a valid tool to assess ECs within the nonhematopoietic compartment.

Collectively, ECs are equipped with the functional machinery to detect LPS and are indeed the dominant G-CSF-producing cell population upon systemic LPS challenge, sufficient to induce emergency granulopoiesis.

EC-intrinsic MYD88 signaling is required to stimulate accelerated neutrophil production, myeloid progenitor lineage skewing toward granulocyte-macrophage progenitors, and increased CFU-G activity in vivo

Next, we assessed neutrophil generation in the BM of *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice in the steady state and during LPS-induced emergency granulopoiesis (Figure 5A). During 12 hours, approximately 10% and 50% of BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils incorporated BrdU into newly synthesized DNA in PBS-injected control and *Tie2-Cre;Myd88^{fl/fl}* mice, respectively (Figure 5B-D). After LPS injection, there was a significant increase in BrdU incorporation in *Myd88^{fl/fl}* mice in both BM CD11b⁺Gr1^{high} mature and BM CD11b⁺Gr1^{low} immature neutrophils. However, this increase was absent in LPS-injected *Tie2-Cre;Myd88^{fl/fl}* mice (Figure 5B-D). These results

demonstrate that neutrophil turnover is significantly accelerated upon LPS stimulation, and that this response is dependent on EC-intrinsic MYD88 signaling.

We also analyzed the myeloid progenitor compartment in steady-state and LPS-injected *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. In accordance with previously published results,⁴⁴ we observed an increase in the percentage of Lin⁺cKit⁺Sca1⁺FcγR⁺CD34⁺ granulocyte-macrophage progenitors (GMPs) upon LPS stimulation in *Myd88^{fl/fl}* mice. This response was absent in *Tie2-Cre;Myd88^{fl/fl}* mice (Figure 5E-F). These data demonstrate that LPS induces a lineage bias at the HPC level in favor of enhanced granulopoiesis.

Finally, we determined the CFU activity in BM from steady-state or LPS-injected *Myd88^{fl/fl}* and *Tie2-Cre;Myd88^{fl/fl}* mice. After LPS injection, there was a significant increase in the overall number of CFUs in *Myd88^{fl/fl}* mice. Notably, this increment was due to an increase in CFU-granulocyte (CFU-G) with no changes in the number of other CFU types. Importantly, this response was absent in LPS-treated *Tie2-Cre;Myd88^{fl/fl}* mice (Figure 5G).

Taken together, these data unambiguously demonstrate that, besides its many known biological effects, including stimulation of hematopoietic stem cell division⁴⁵ and of neutrophil migration and recruitment to inflamed tissues,^{14,15} LPS stimulates emergency granulopoiesis via MYD88 signaling in ECs.

EC-intrinsic MYD88 signaling is required to efficiently stimulate emergency granulopoiesis during systemic *E. coli* infection

To evaluate whether MYD88 signaling in ECs is also important to initiating emergency granulopoiesis in a far more complex setting of infection with living bacteria, we performed experiments with

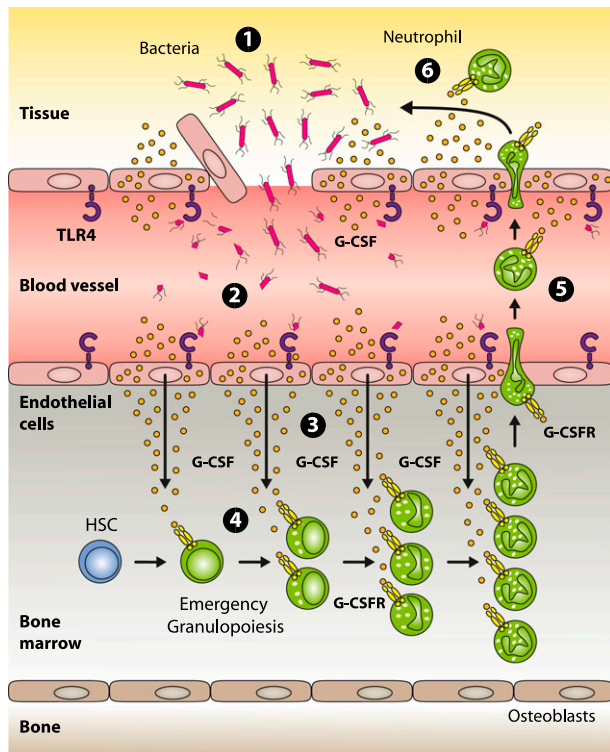


Figure 7. Model for pathogen sensing and subsequent translation into emergency granulopoiesis. (1) Gram-negative bacteria and/or their structural components that have gained access to the systemic circulation are recognized by TLR4-expressing endothelial cells (2), thereby indicating an emergency state. Upon TLR4/MYD88 signaling in endothelial cells, G-CSF is released in large quantities (3). In the bone marrow, endothelial cell-derived G-CSF acts on myeloid precursors expressing the G-CSF receptor resulting in enhanced generation, accelerated turnover, and increased neutrophil release from the bone marrow to the systemic circulation (4). These neutrophils are recruited to the site of infection (5) where they participate in clearing the pathogen (6).

E. coli. First, we titrated the *E. coli* dose necessary to elicit a similar quantitative response in WT mice as observed with the LPS dose used in prior experiments. We determined this dose to be 4.5×10^8 *E. coli* CFU given IP per WT mouse (supplemental Figure 3A-E). In parallel, we also infected *Myd88*^{-/-} mice. Although these mice showed some response to *E. coli*, the responses were severely and highly significantly reduced compared with WT mice for the entire previously assessed readouts characteristic for emergency granulopoiesis (supplemental Figure 3A-E). Importantly, the minor MYD88-independent responses upon *E. coli* infection are also independent of G-CSF because there was no significant rise in plasma G-CSF levels in *Myd88*^{-/-} mice (supplemental Figure 3E).

Next, we infected control *Myd88*^{fl/fl} and *Tie2-Cre;Myd88*^{fl/fl} mice with *E. coli* (4.5×10^8 CFU per mouse). We observed the same pattern, with *Myd88*^{fl/fl} mice showing the same response as WT mice, and *Tie2-Cre;Myd88*^{fl/fl} mice showing a minor, in comparison with *Myd88*^{fl/fl} mice significantly reduced response. This indicates that EC-intrinsic MYD88 signaling is required to induce full-blown emergency granulopoiesis also upon live *E. coli* infection (Figure 6A-E). The minor response observed in *Tie2-Cre;Myd88*^{fl/fl} mice in an infection with live bacteria might not be surprising because in this far more complex situation, in contrast to exclusive LPS-mediated stimulation, other pattern-recognition receptor agonists and additional tissue damage also might activate alternative, independent signaling pathways. Notably, however, plasma G-CSF levels did not rise above steady-state levels in *Tie2-Cre;Myd88*^{fl/fl} mice, revealing ECs to be major sources of G-CSF also during infection with *E. coli* (Figure 6E).

To test whether TLR adaptor TRIF might partially compensate for the severely diminished *E. coli*-induced emergency granulopoiesis in *Myd88*^{-/-} and *Tie2-Cre;Myd88*^{fl/fl} mice, and to determine the relative contribution of each pathway to it, we infected WT, *Tlr4*^{-/-}, *Myd88*^{-/-}, and *Trif*^{-/-} mice with 4.5×10^8 *E. coli* CFU IP. We observed that both *Tlr4*^{-/-} and *Myd88*^{-/-} mice show an equally reduced ability to launch emergency granulopoiesis compared with WT mice. By stark contrast, *Trif*^{-/-} mice responded to *E. coli* (supplemental Figure 4) with emergency granulopoiesis, as did WT mice. These data demonstrate that TRIF is dispensable for the initiation of emergency granulopoiesis.

In summary, our findings demonstrate that abrogated MYD88 signaling in ECs leads to severely defective emergency granulopoiesis upon *E. coli* infection.

Discussion

We addressed here the fundamental question of which cell types act as primary sensors of bacterial dissemination during severe infection and consequently induce the switch from steady-state to demand-adapted emergency granulopoiesis.

Two general models have been proposed for how pathogen sensing and translation into emergency granulopoiesis may be achieved.^{7,8} According to the concept of indirect hematopoietic activation, tissue-resident macrophages might serve in this critical function^{46,47} because they indeed express pattern-recognition receptors, and, after respective stimulation, produce granulopoietic growth factors.⁴⁸⁻⁵¹ But data from stringent in vivo experimentation supporting this model are lacking. Alternatively, several recent studies suggested a model of direct hematopoietic activation based on the observation that mouse and human HSPCs express TLRs and that TLR agonist stimulation leads to enhanced myelopoiesis.^{16-19,21} However, these studies used either in vitro¹⁶ or very specific in vivo experiments such as adoptive HSPC transfer under the renal capsule,¹⁸ direct injection of HSPCs into *Staphylococcus aureus*-infected wounds,²¹ or pre-treatment with chemotherapeutic agents and ionizing radiation²² to convey their respective proposed model. By contrast, we have demonstrated that pathogen sensing by both immature HSPCs and mature hematopoietic cells, including macrophages, is dispensable for the acute process of emergency granulopoiesis in 3 independent and complementary experimental approaches. First, in both hematopoietic *Tlr4*^{-/-} chimeric mice²³ and hematopoietic *Myd88*^{-/-} chimeric mice, emergency granulopoiesis is indistinguishable from that in WT mice. Of note, we and others could show that BM chimeric mice are an appropriate tool to broadly dissect biological effects contributed by either hematopoietic or nonhematopoietic tissues by confirming that tissue-resident macrophages, although they are able to locally self-renew in the steady state,⁵² are replenished by donor-derived cells after lethal irradiation.^{23,52} Second, liposomal clodronate-mediated macrophage-depleted WT mice launch undiminished emergency granulopoiesis upon LPS stimulation.²³ Third, as shown here, mice with a *Myd88*-deficiency in the myeloid compartment, most importantly macrophages (*LysM-Cre;Myd88*^{fl/fl} mice), respond normally toward LPS with emergency granulopoiesis.

To determine the identity of the nonhematopoietic cell types required for the initiation of emergency granulopoiesis, we undertook an extensive genetic targeting approach using Cre recombinase-mediated tissue-specific *Myd88* ablation. Specifically, we targeted the nonhematopoietic BM microenvironment (*Nestin*⁺ mesenchymal stem cells and perivascular stromal cells) using *Nes-Cre*³⁶⁻³⁸ and *Pdgfrb-Cre*³⁹

mice. These cells have been shown to be part of the hematopoiesis-supporting BM microenvironment.⁵³ Vascular cells (ECs and pericytes) were targeted using *Tie2-Cre*^{37,38,40,54} and *Pdgfrb-Cre*³⁹ mice, respectively. In addition, we used *Alb-Cre*⁴¹ mice to target hepatocytes as a candidate prototypic parenchymal cell type. Our data revealed that ECs express high amounts of *Tlr4* and *Myd88* and respond to systemic LPS stimulation or *E coli* infection with massive upregulation of the primary granulopoiesis-supporting growth factor G-CSF. Most importantly, mice with a *Myd88* deficiency in ECs do not respond with emergency granulopoiesis upon systemic LPS stimulation and have a severely defective response toward *E coli* infection. The *E coli*-driven MYD88- and G-CSF-independent minor responses in *Myd88*^{-/-} and *Tie2-Cre;Myd88*^{fl/fl} mice are likely triggered by activation of alternative, redundant pathways that evolved to secure this critical response. Of interest, a recent study showed that granulopoiesis after antibody-mediated neutrophil removal depends on TLR4/TRIF signaling.⁵⁵ We therefore studied whether a similar feedback regulatory mechanism via TLR4/TRIF might account for the residual emergency granulopoiesis observed in *E coli*-infected *Myd88*^{-/-} and *Tie2-Cre;Myd88*^{fl/fl} mice. However, we observed that TLR4 signal transduction via TRIF is dispensable for the induction of *E coli*-driven emergency granulopoiesis as *Trif*^{-/-} mice show a response that is indistinguishable from those in WT mice.

Because congenital³³ or iatrogenic³² forms of neutropenia are associated with a high incidence of lethal infectious complications, it seems very likely that the ability to induce emergency granulopoiesis per se has a live-saving role in massive infection. However, direct experimental evidence for this notion is lacking and future studies will need to dissect emergency granulopoiesis from neutrophil recruitment to inflamed tissues and neutrophil effector functions, which together ensure host survival during bacterial infection.

Given the general caveat of lack of absolute tissue specificity in the *Cre-loxP* system, we have analyzed *Tie2-Cre-loxP-GFP* reporter mice. In accordance with the literature,^{37,38,40,54} *Tie2-Cre* mice are an excellent tool to genetically manipulate ECs, but, of note, there is also a small fraction of other (ie, non-EC and nonhematopoietic) cells targeted in *Tie2-Cre* mice. However, we have addressed this issue experimentally by analyzing the G-CSF response in non-ECs in these mice and could exclude a relevant contribution of these nonhematopoietic, non-EC cell populations to the overall emergency granulopoiesis response.

Interestingly, the versatile and critical function of endothelium is an emerging aspect in hematopoiesis research. It has recently been demonstrated that ECs are an important constituent of the BM microenvironment. Using a similar genetic tissue-specific deletion approach, ECs were shown to be major sources of stem cell factor³⁷ and CXCL12,^{38,54} 2 molecules that are involved in HSPC maintenance and their BM retention, respectively. Our results now show that ECs are not only critical for the regulation of steady-state

hematopoietic maintenance but are also essential for demand-adapted hematopoiesis in response to the paradigmatic gram-negative bacteria-derived compound LPS and the clinically relevant pathogen *E coli*. In conjunction with previously published data on the importance of TLR signaling in ECs for neutrophil recruitment to inflamed tissues,^{14,15} our findings reveal a sophisticated degree of functional interplay between the vascular, the hematopoietic, and the immune systems, which have a common developmental root and might thus be understood as 1 functional organ.^{56,57} Of note, our findings also confirm and extend prior landmark observations on the importance of nonhematopoietic cell-derived hematopoietic growth factors.^{58,59} Although these seminal studies established a role for nonhematopoietic cells as a major source of hematopoietic growth factors, they, because of technical limitations at that time, were unable to identify the precise identity of this cell type that we now show to be ECs.

ECs are ideally positioned to mark the threshold of local vs systemic infection, and using ECs as safeguards seems a very plausible, danger-adapted defense strategy established in evolution. In summary, based on the data presented here, we propose a model (Figure 7) in which ECs are critical for pathogen sensing and subsequent induction of emergency granulopoiesis.

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Authorship

Contribution: S.B. devised, performed and analyzed experiments, and wrote the manuscript; R.C.G., R.R., J.B., and F.A. performed experiments; M.H. and M.K. devised experiments and discussed data; and M.G.M. directed the study and wrote the manuscript.

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